Exhibit 17

Formation of protoporphyrin IX in mouse skin after topical application of 5-aminolevulinic acid and its methyl ester.

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ABSTRACT

Normal skin of nude mice (Balb/c) was treated topically with 5-aminolevulinic acid (ALA) and its methyl ester (ALA-Me) for 24 hours. Approximately 0.1 gram of freshly prepared cream was applied to a spot of 1 cm^2 on the flank of the mice, which was then covered with a transparent dressing. The ALA induced protoporphyrin IX (PpIX) was studied by means of a noninvasive fibre-optic fluorescence probe connected to a luminescence spectrometer. The excitation wavelength was 407 nm, and the emission wavelength was 637 nm.

For the first hour a slight lag in PpIX production was observed for the mice treated with ALA-Me compared to the mice treated with ALA. After approximately 12 hours the ALA and the ALA-Me treated mice showed the same PpIX fluorescence intensity. From 12 hours until 24 hours the PpIX fluorescence intensity decreased for both treatment modalities, even though ALA and ALA-Me were continuously present. At 24 hours ALA-Me-treated mice had less than half the amount of PpIX in their skin compared with ALA-treated mice.

Keywords: *Photodynamic therapy, Fluorescence monitoring, Prodrug, 5-aminolevulinic acid (ALA).*

1. INTRODUCTION

A substantial amount of clinical work is being carried out with 5-aminolevulinic acid (ALA)-generated protoporhyrin IX (PpIX) as sensitizer, and photodynamic therapy (PDT) with ALA has been shown to be a promising treatment for superficial skin malignancies. A limited depth of ALA penetration through the skin significantly limits the usefulness of this modality[1,2]. Basal cell carcinoma (BCC) is the most frequent malignant tumour in the white population. For nodular BCC, in contrast to superficial BCC, PDT based on topical application of ALA has a low complete response rate[2,3]. Human skin is poorly permeable to most foreign substances, including drugs. Thus, methods to increase the penetration of ALA into all layers of the tumour are crucial for a more extensive use of the ALA PDT modality.

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ALA is a hydrophilic molecule, and does not easily penetrate biological barriers with lipophilic properties such as the stratum corneum of the skin or cell membranes[4]. Dermal delivery may be improved by derivatisation of ALA. More lipophilic ALA derivatives, like ALA esters, are expected to penetrate deeper into the tissue than ALA itself. An improvement of the bioavailability of ALA by use of an ALA-prodrug is related to two processes: The rate of diffusion of the prodrug through biological barriers and the rate of enzymatic conversion into the parent ALA[5].

An important advantage of using ALA or some of its derivatives instead of other photosensitizers like hematoporphyrin derivative or phthalocyanines, is that the photosensitizing effect of the ALA-induced PpIX on skin does not remain much longer than 48 hours and thus the patients do not suffer from prolonged skin photosensitivity[6]. PpIX also accumulates in tumour tissue to a greater extent than most other photosensitizers, minimising damage to normal surrounding tissues during treatment[1,7].

It is possible to study ALA induced PpIX kinetics by means of a noninvasive fibre-optic fluorescence probe connected to a luminescence spectrometer[8]. In the present work we have studied PpIX kinetics in nude mouse skin, generated by topical application of ALA or its methyl ester (ALA-Me).

2. MATERIALS AND METHODS

Animals. Normal female Balb/c nude mice, approximately 6-12 weeks old, were used. Typically, the mice weighed 25 gram. Food and water was provided ad libitum. The animals were anaesthetized with hypnorm/dormicum (approximately 4 ml/kg body weight) 10 minutes prior to the application of the cream. No anaesthetics were used during the measurements.

Chemicals. The ALA and the ALA-Me hydrochlorides were obtained from Sigma (St. Louis, MO). The ALA cream (20% w/w) was made by dissolving 200 mg ALA hydrochloride in 200 mg distilled water, and then mixing this solution into 600 mg of a cream base (Unguentum, Merck, Germany). The ALA-Me cream was made in the same way, but to obtain the same molar amount of ALA as in the ALA cream, more of the ALA-Me hydrochloride (216 mg) and less distilled water (184 mg) were used. Approximately 0.1 gram of freshly prepared cream was applied to a 1 cm^2 spot on one flank of the mice, and then covered with a transparent dressing (OpSite Flexigrid, Smith & Nephew Medical Ltd., England). During the application of the cream, the skin fluorescence was measured every hour for the first 6 hours, and then every second hour until 14 hours. The fluorescence was also measured 24 hours after application of the cream. The transparent dressing was kept on during the entire experiment, and did not disturb the measurements.

Fluorescence detection. During the measurements the mice were not anaesthetized, but carefully held onto a table. The ALA-induced PpIX fluorescence was detected by means of a noninvasive fibre-optic fluorescence probe connected to a luminescence spectrometer (Perkin Elmer LS50B). The excitation wavelength was 407 nm, and the emission wavelength was 637 nm.

3. RESULTS AND DISCUSSION

Juzenas *et al* (manuscript in preparation) have found that penetration of ALA and ALA-Me into the skin is a relatively fast process while the biosynthesis of PpIX is significantly slower, taking several hours to yield the maximal amount of PpIX. For the first hour a slight lag in PpIX production was observed for the mice treated with ALA-Me compared to the mice treated with ALA (figure 1). According to Kloek *et al.*[5] this could be due to the fact that the ALA ester needs some time to be converted into ALA before it can be used in the heme synthetic pathway. After approximately 12 hours the ALA and the ALA-Me-treated mice showed the same PpIX fluorescence intensity. This is in good agreement with Kloek *et al.*[5] who have shown (in vitro) that the difference between ALA and its esters with respect to PpIX production is less pronounced after such incubation periods.

From 12 hours until 24 hours the PpIX fluorescence intensity decreased for both treatment modalities, even though ALA and ALA-Me were continuously present. At 24 hours ALA-Me-treated mice had less than half the amount of PpIX in their skin compared with ALA-treated mice. This indicates faster clearence of the ALA-Me than the ALA, and thus an even shorter duration of the skin photosensitivity.

We also observed a systemic effect in the mice 6-8 hours after starting topical application of the 20% ALA cream; i.e. the skin of the whole mouse was fluorescing when illuminated with a UV lamp (figure 2a). On the other hand, even after 24 hours of continuously topical application of ALA-Me cream, PpIX fluorescence could be found only in the spot where the ALA-Me cream had been applied (figure 2b). Studies in humans also indicate that ALA-Me accumulates more selectively in the lesions than ALA[9]. In addition Peng *et al.*[10] have found ALA ester-induced porphyrin fluorescence in human nodular BBC lesions to be more homogenously distributed than the PpIX fluorescence induced by ALA.

4. ACKNOWLEDGEMENTS

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5. REFERENCES

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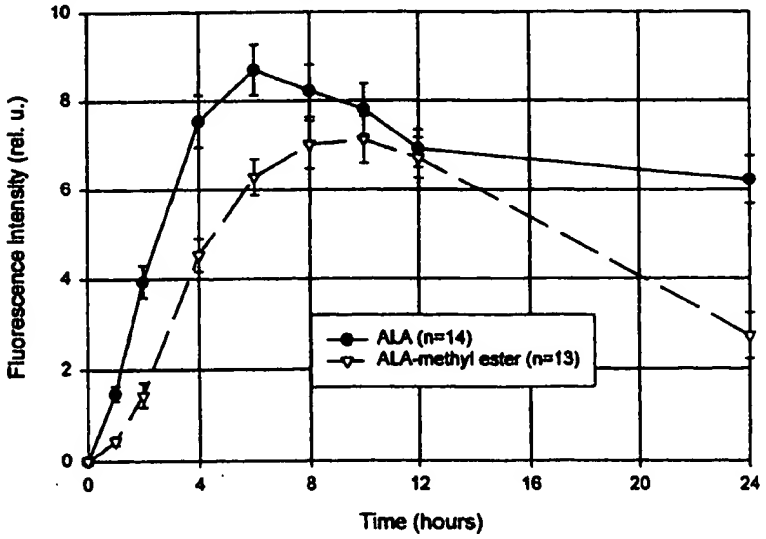

Figure 1: Mean initial PpIX fluorescence as a function of time after topical application of ALA and ALA-methyl ester on normal nude mice skin.

Figure 2: PpIX fluorescence in Balb/c nude mice skin after continuously topical application of a) 20% ALA cream and b) 20% ALA-methyl ester cream for 8 hours. The mouse in picture a) shows fluorescence all over, while mouse b) shows fluorescence only in the spot where the cream was applied. The mice where illuminated by a UV lamp during the photo session.